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The Use of Primer-Specific Targeting on Mitochondrial Cytochrome b Combined with Real-Time Polymerase Chain Reaction for the Analysis of Dog Meat in Meatballs

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Highlights

- Cyt-55 primer has been designed for real-time polymerase chain reaction for detection of dog meat in meatballs.
- Cyt b-55 could specifically amplify DNA from dog meat using an optimum annealing temperature of 57.9°C.
- Real-time PCR using Cyt b-55 primer could be proposed as a standard method for the identification of dog meat in food products for halal authentication study.

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The Use of Primer-Specific Targeting on Mitochondrial Cytochrome b Combined with Real-Time Polymerase Chain Reaction for the Analysis of Dog Meat in Meatballs

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Abstract: The adulteration of beef with lower price meat such as dog meat is common to obtain an economic profit. Dog meat is non-halal for Islamic followers. This study was intended to design primer-specific targeting of the mitochondrial cytochrome b gene for the detection of DNA extracted from dog meat in meatball products. The results showed that Cyt b-55 could specifically amplify DNA from dog meat using an optimum annealing temperature of 57.9°C. Real-time polymerase chain reaction (PCR) using Cyt-55 primer could detect the presence of DNA at a concentration as low as 0.25 ng/mL, corresponding to 1% of dog meat in beef meatballs. The efficiency (E) values obtained were 91.2% and 110.8% for amplification using DNA extracted from fresh dog meat and dog meat in meatballs, respectively. The repeatability of the real-time PCR method was reliable, as indicated by the low value of relative standard deviation of cycle threshold (Ct) values from 6 replicates, namely, 0.91% (from DNA extracted from fresh meat) and 1.09% (from DNA extracted from meatballs). Real-time PCR using Cyt b-55 primer could be proposed as a standard method for the identification of dog meat in food products to ensure that they are halal and pure.

Keywords: Dog Meat, Cyt b-55 Primer, Meatball, Halal Authentication, Polymerase Chain Reaction.

INTRODUCTION

The global halal market has great potential to increase in future years, driven by the development of halal ingredient authentication methods that strengthen consumers' confidence (Hameed *et al.* 2018). In recent years, there has been an increasing focus among Muslim consumers on consuming halal food, which is free from any prohibited components, such as dog meat. As a consequence, there is a need for

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reliable and accurate information about meat composition in food products (Ballin 2010; Rohman *et al*. 2011). Meat is considered to be a good source of protein, and among food commodities, meat is highly subjected to adulteration. The adulteration of meat products occurs through the partial or total substitution of high-priced meat with lower price meat. The practice of meat adulteration poses some religious and economic problems. From a religious point of view, meat adulteration with non-halal meat such as dog meat is a serious matter because dog meat is not allowed to be consumed by Muslim communities (Montowska & Pospiech 2011; Ulca *et al.* 2013). From an economic perspective, the adulteration of halal-meat with non-halal meat can result in an economic profit for food producers due to the price difference between halal and non-halal meat; therefore, some unethical producers try to substitute halal meat with non-halal meat (Sahilah *et al.* 2012). This issue has encouraged scientists to develop analytical methods capable of detecting the presence of non-halal meat.

The identification of meat species presents in food products including meatballs is of great importance in order to assess the authenticity of meat-based food products, because processing frequently makes food difficult to distinguish in terms of meat composition. For the sake of authentication purposes, numerous analytical techniques based on physiochemical and biological properties have been reported as analytical tools for meat identification in a variety of meat-based foods based on the identification of specific markers targeting lipid, DNA or protein in meat (Mafra *et al.* 2008; Abbas *et al.* 2018). Analytical methods used for halal meat authentication based on lipid analysis include Fourier-transform infrared (FTIR) spectroscopy (Rohman *et al.* 2011; Kurniawati *et al*. 2014), two-dimensional gas chromatography (GC x GC) (Indrasti *et al*. 2010), electronic nose and gas chromatography-mass spectrometry (Nurjuliana *et al.* 2011), and differential scanning calorimetry (Mansor *et al.* 2012). Protein-based methods include immunoassays (Ghovvati *et al.* 2009), electrophoresis (Vallejo-Cordoba *et al.* 2010), chromatography, mass spectrometry (MS) and spectroscopy (Montowska & Pospiech 2011). Chromatography in combination with mass detection is suitable for the analysis of specific markers in meats, but these methods are costly, which leads to DNA-based methods being a preferred method for the identification of meat species.

DNA-based methods using polymerase chain reaction (PCR) offer highly specific, fast, sensitive and less costly alternatives for the identification of meat species even in complex processed foods (Amaral *et al*. 2015; Bottero & Dalmasso 2011). Numerous approaches for PCR including restriction fragment length polymorphism (RFLP-PCR) (Aida *et al*. 2005), specific PCR (Che Man *et al*. 2007), multiplex PCR (Ali *et al*. 2015), PCR-southern hybridization (Mutalib *et al*. 2015) and loop-mediated isothermal amplification (Ran *et al*. 2016) have been proposed and used for the identification of meat for halal authentication purposes.

Real-time PCR using specific primers with fluorescent probes such as SYBR Green has been used for meat species identification, especially for identification of non-halal meat, such as pork, wild boar meat, and rat meat in several food products including meatballs, dendeng and abon (Widyasari *et al*.

2015; Maryam *et al*. 2016; Rahmawati *et al*. 2016; Guntarti *et al*. 2017) as well as porcine gelatine-containing products (Sudjadi *et al.* 2016). In this study, a species-specific primer targeting the mitochondrial cytochrome b gene in combination with real-time PCR has been used for the detection of dog DNA in meatball products. The cytochrome b gene was chosen because it revealed an appropriate degree of intra- and interspecies variability. In addition, this gene also offers a high number of copies per cell, which increases the sensitivity of real-time assay significantly and contributes to the survival of copies of DNA when tissue has been subjected to extreme processing conditions, for example sterilization and boiling (Girish *et al.* 2004).

MATERIALS AND METHODS

Meats used in this study, namely, dog meat, chicken, beef and goat, were obtained from local markets and slaughter houses in Yogyakarta, Indonesia. Monkey meat was supplied by the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta. Numerous samples of meatballs were obtained from local markets around Yogyakarta. The designed specific primer was purchased from Genetica Science (Jakarta, Indonesia).

Primer Design

The specific primer targeting mitochondrial cytochrome-b (myt cyt-b) was designed by software provided by Integrated DNA Technologies (IDT, California, US). Both forward and reverse primers were subjected to Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The primer Cyt b-55 designed was as follows:

Forward Primer: AGCCATGCACTACACATCAGA
Reverse Primer: CCGTAACTGACGTCTTGACA

Preparation of Laboratory-Made Meatballs

For preparation of meatballs with a known composition of meat, beef and dog meat were mixed to obtain beef-dog meat ratios of 0:100, 1:99, 5:95, 10:90, 25:75, 50:50, 75:25 and 100:0. The meatballs were made by mixing 90% fresh meat with other components (10%) including tapioca starch and selected spices, such as garlic and cooking salt. The meatball components were subjected to emulsification and made into ball shapes manually. The meatballs made were introduced into boiling water (Purnomo & Rahardiyan 2008).

DNA Extraction

The procedure of DNA extraction in meatballs and fresh meat was carried out according to Sambrook and Russell (2001). Fresh meat samples as well as laboratory-made and commercial meatballs were cut into small pieces and ground with mortar and pestle. Ground samples (200 mg) were combined with 1000 µL of lysis buffer comprising Tris HCl, EDTA, NaCl and SDS 1%, combined with 30 µL proteinase K (20 mg/mL) and then vortexed for 5 min. The mixture was incubated at 55°C for 1 h and then centrifuged at 13.000 rpm for 15 min. In a new microtube, the supernatant was combined with cold phenol (0.5 × volume) and shaken for 30 min with a shaker, followed by centrifugation at 13.000 rpm for 10 min. The supernatant was transferred into a new microtube, combined with chloroform (0.5 × volume), homogenized and centrifuged at 13.000 rpm for 10 min. The supernatant in a new microtube was combined with Na-acetate 3M pH 5.2 (0.1 × volume) and absolute ethanol (2 × volume), and incubated at –4°C overnight. The pellet containing DNA was subsequently washed with 250 µL ethanol 70%, and dissolved in 50 µL TE buffer. The DNA obtained was stored at –20°C for further analysis.

Qualitative Analysis and Purity Evaluation of DNA

Qualitative analysis of DNA obtained during this isolation was performed using electrophoresis gel agarose with a concentration of gel agarose of 0.8% using TBE buffer. DNA was stained using *GelRed®.* Electrophoresis was performed using a voltage of 100 V for 60 min, and its results were visualised using UV-transilluminator (Sambrook & Russell 2001). The purity and concentration of DNA was determined by measuring its absorbance value of 2 µL isolate (in TNE buffer) containing DNA using NanoVue® Plus Spectrophotometer at wavelengths of 230, 260 and 280 nm to obtain the purity and concentration of evaluated DNA.

$$\text{[DNA] (ng/ µL)} = A_{260} \times \text{dilution factor} \times 50 \text{ ng/µL}$$
$$\text{Purity index} = A_{260} / A_{280}$$

Analysis Using Real-Time PCR

Analysis using real-time PCR was carried out with a PCR CFX96 instrument (Biorad, USA) using a total volume of 20 µL, which consisted of 1 µL SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 1 µL forward primer 10 µM, and 1 µL reverse primer 10 µM, with each primer having a final concentration of 500 nM, 1 µL DNA template (50 µg/mL), and 7 µL nuclease free water. The PCR thermocycler was programmed as follows: predenaturation at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 sec; the annealing temperature was optimised at 50°C–62°C for 10 sec based on Tm of primer, with elongation at 72°C for 30 sec. The positive control (laboratory-made meatballs), commercial

samples and NTC (no template control) were run in triplicates. Melting curve analysis was carried out at 65°C–95°C with a slope of 0.5°C/sec.

Validation of Real-Time PCR

Validation of real-time PCR analysis was carried out by assessing numerous performance characteristics, including the specificity of designed primer, sensitivity expressed with detection limit, and precision evaluated by repeatability test. The method validated was subsequently used for analysis of commercial meatballs. The sensitivity of real-time PCR using designed primer was expressed by the limit of detection (LoD). The LoD value was the lowest amount of DNA that could be amplified with a reproducible cycle threshold (Ct) value (Sudjadi *et al.* 2016). The LoD evaluation was carried out by making a dilution series of DNA extracted from dog meat at concentrations of 50,000; 25,000; 12,500; 6,250; 3,125; 1,562.5 and 781.25 pg of DNA.

RESULTS AND DISCUSSION

The first step for identification of the species origin of the meat contained in the meatball products was the extraction of DNA. The concentration and purity of extracted DNA in the fresh meat and in the meatballs were assessed by measuring isolate containing DNA at wavelengths of 280 and 260 nm, which are shown in Tables 1 and 2. The purity index of all DNA isolates in the fresh meat and meatballs ranged from 1.765 to 1.961 and 1.804–1.986, respectively. The high purity of the DNA extracts in the raw meat compared to those of the reference meatballs suggested that the heat processing did not affect the quality of DNA.

Table 1: Concentration and purity of DNA extracted from fresh meat.

Fresh meat	Concentration (ng/µL)	A_{260}	A_{280}	Purity index
Dog meat	831	16.60	7.63	1.845
Chicken	3416	69.36	35.88	1.961
Goat	1264	26.68	15.72	1.765
Wild boar	2561	51.90	27.62	1.901
Beef	2182	44.50	23.76	1.906
Pork	783.5	16.10	8.66	1.904
Monkey meat	2603	53.02	28.40	1.897

The designed primer (Cyt b-55) has a melting temperature of 59.44°C for the forward primer and 57.59°C for the reverse primer with amplicon length of 75. The first step for analysis of DNA from dog meat was the optimisation of annealing temperature, with the best amplification provided by setting an annealing

temperature of 50.4°C–59.4°C. Primer Cyt b-55 was capable of providing the high amplification response of dog meat DNA, with a relative fluorescent unit (RFU) of 1,366, an annealing temperature of 57.9°C (Fig. 1A) and a quantification cycle (Cq) of 27.40, with a melting temperature (Tm) of 77.50 (Fig. 1B).

Table 2. Concentration and purity of DNA extracted from the reference meatballs**.**

Type of sample	Amount of dog meat (%)	Amount of beef (%)	Concentration (ng/µL)	A260	A280	Purity index (A_{260}/A_{280})
BP 1	100	0	652	13.41	7.39	1.858
BP 2	75	25	1,026	20.28	11.06	1.818
BP 3	50	50	936	19.13	10.49	1.857
BP 4	25	75	762	15.80	8.69	1.875
BP 5	10	90	1,006	22.08	12.85	1.847
BP 6	5	95	917	19.54	11.24	1.827
BP 7	1	99	557	15.31	9.78	1.986
BP 8	0	100	1,010	21.96	12.96	1.804

Note: BP = reference meatballs (meatballs prepared in the laboratory)

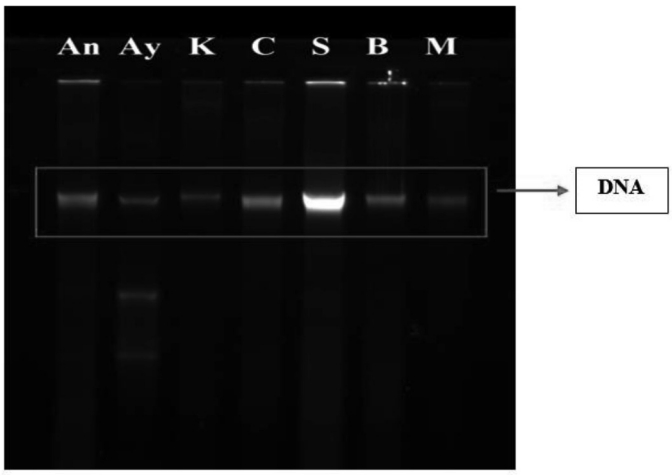
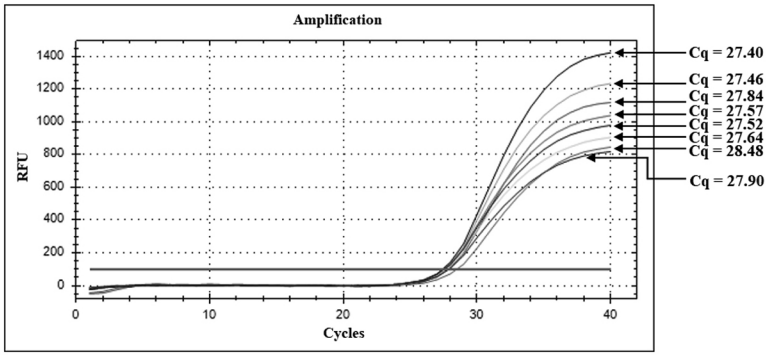


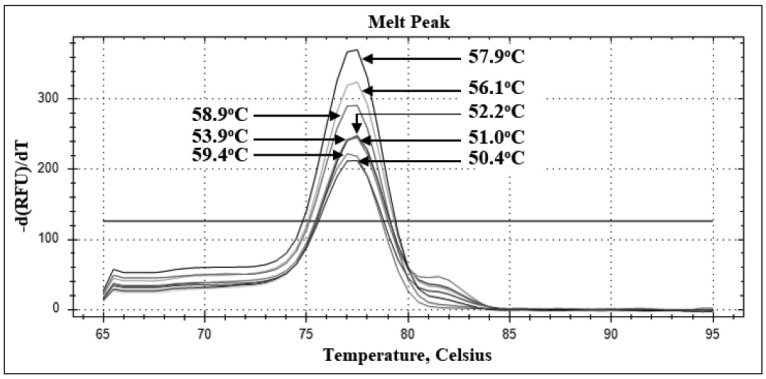
Figure 1: Electrophoresis results of DNA isolates from several meats: dog (An), chicken (Ay), goat (K), wild boar (C), beef (S), pork (B), and monkey (M) on agarose gel 0.8%.

For quantitative analysis purposes, the primer of Cytb-55 was validated by determining several performance characteristics, which included the specificity, linearity, efficiency, limit of detection for expression of sensitivity, and precision as determined using a repeatability test according to Bustin *et al*. (2009). The primer specificity was evaluated by amplifying DNA extracted from several meats of *Sus scrofa* (pork), *Bos taurus* (beef), *Capra hircus* (sheep), *Sus scrofa domesticus*

(wild boar), *Gallus gallus* (chicken) and *Macaca fascicularis* (monkey). Fig. 2 shows the result of the specificity test in which the primer Cyt b-55 only amplified DNA from dog meat, indicating that the designed primer was specific to other DNA from meat commonly used in preparation of meatball products. Specificity is very important and is the only parameter needed to be validated during qualitative and confirmation analyses as required by the Association of Official Analytical Chemists (AOAC) (ISO, 2017).

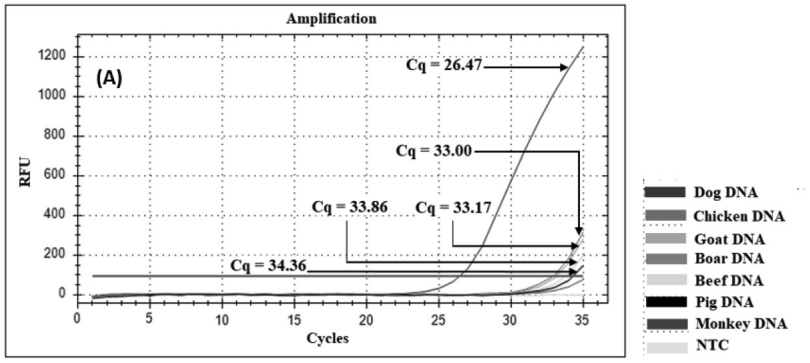


(A)

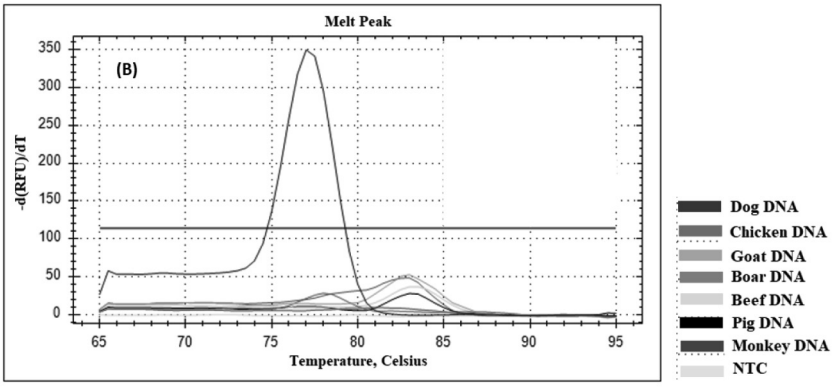


(B)

Figure 2: Optimisation of annealing temperature using Primer Cyt b-55 for DNA extracted from dog meat. (A) Amplification curve and (B) melting curve analysis.



(A)

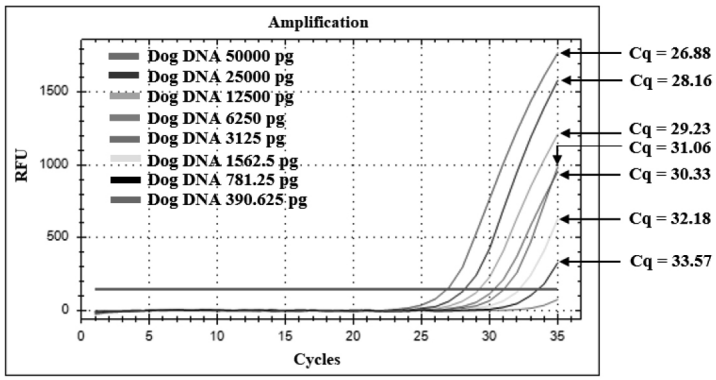


(B)

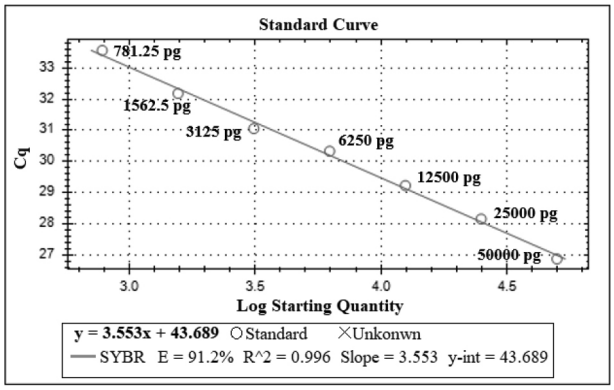
Figure 3: Specificity test of primer Cyt b-55 towards DNA extracted from several meats using optimum annealing temperature of 57.9°C. (A) Amplification curve and (B) melting curve during amplification.

The identification of dog meat can be considered as determining whether a product is halal because its presence even in very low concentrations is not allowed. Halal products are zero tolerance; therefore, a determination of sensitivity to know the detection limit is necessary. The sensitivity of analytical methods is typically expressed by the limit of detection, which can be understood as the lowest concentration of DNA detected in samples. In this study, two detection limits, relative and absolute detection limits, were determined. The absolute detection limit was assessed by diluting stock DNA to obtain the serial concentration of DNA at a certain dynamic range covering 5,000–390 ng. The LoD was determined through linear regression of a logarithm of DNA concentration extracted from dog meat (x-axis) and quantification cycle value (Cq) (y-axis). The results in Fig. 4

showed that the absolute LoD for dog meat DNA was 390 pg, considering that at 390 pg, the amplification occurred. For determination of the relative LoD, the percentage of dog meat added to the meatballs made in the laboratory were at 1%, 5%, 10%, 20%, 50%, 75% and 100%, with the remaining meat being beef. The obtained relative LoD was 1%, based on the fact that at < 1%, linearity was no longer suitable for the acceptance criteria of real-time PCR assay (Fig. 5).

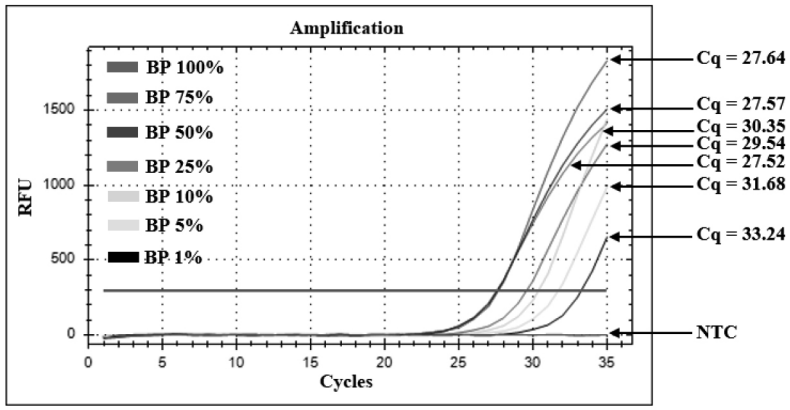


(A)

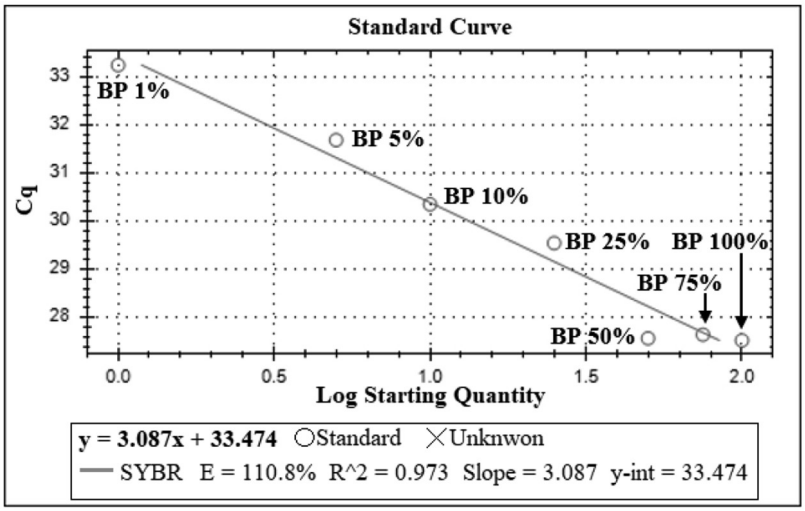


(B)

Figure 4: Sensitivity results for determination of the absolute LoD in terms of the Cq values of primer Cyt b-55 used for amplification of DNA extracted from fresh dog meat with different concentrations (A) along with standard curve correlating between log concentration (x-axis) and Cq values (y-axis).



(A)



(B)

Figure 5: Determination of the relative LoD in terms of the Cq values of primer Cyt b-55 used for amplification of DNA extracted from meatballs with different amounts of dog meat (A) along with standard curve correlating between log concentration (x-axis) and Cq values (y-axis).

The efficiency (E) values of primer Cyt b-55 for the amplification of the DNA template were determined by constructing a linear regression of Cq values against a logarithm of DNA concentration using DNA extracted from fresh dog meat (Fig. 4B) and from meat formulation (Fig. 5B). The coefficients of determination (R^2) values obtained were 0.996 and 0.973 for DNA extracted from fresh dog meat and meatballs, respectively. The E values obtained were 91.2% for DNA extracted

from fresh meat and 110.8% for DNA extracted from meatballs. For real time-PCR analysis, the acceptable E values were in the range of 90%–110%, indicating that E values obtained during this study met the requirements (Broeders *et al*. 2014). The precision was evaluated by intra-assay and inter-assay using three day assay variation, and relative standard deviation (RSD) values of Cq were used for precision evaluation. RSD values for intra-assay and inter-assay were 0.91% and 1.09%, respectively. The European network of GMO laboratories (ENGL) sets the acceptance criteria for RSD at ≤ 25%; therefore, the obtained RSD values met the acceptance criteria for precision (European Network of GMO Laboratories [ENGL] 2005).

The validated real-time PCR method using primer Cyt b-55 was applied to identification of commercial meatball samples (15 samples) along with a positive control of meatball samples with 100% dog meat and a negative control (no template control, NTC of the meatball with 100% beef). Fig. 6 shows the amplification results in which commercial samples and the NTC did not exhibit any amplification, while the positive control was amplified at Cq of 27.07. This result indicated that the evaluated commercial meatball samples did not contain dog meat.

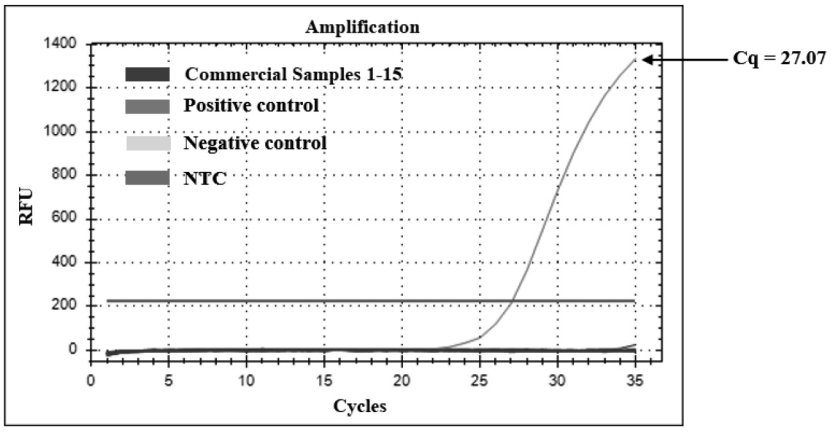


Figure 6: Real-time PCR amplification of DNA extracted from several meatball samples obtained from Yogyakarta, Indonesia.

CONCLUSION

Primer Cyt b-55 in combination with real-time PCR is successfully validated for the identification and quantification of DNA from dog meat in meatball products. The efficiency values and RSD values were acceptable during validation of real-time PCR. Real-time PCR using Cyt b-55 can be proposed as a standard method for the identification of dog meat in meatball products.

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